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Antifungal Activity of Tamoxifen: In Vitro and In Vivo Activities and Mechanistic Characterization▽†

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Tamoxifen (TAM), an estrogen receptor antagonist used primarily to treat breast cancer, has well-recognized antifungal properties, but the activity of TAM has not been fully characterized using standardized (i.e., CLSI) in vitro susceptibility testing, nor has it been demonstrated in an in vivo model of fungal infection. In addition, its mechanism of action remains to be clearly defined at the molecular level. Here, we report that TAM displays in vitro activity (MIC, 8 to 64 μg/ml) against pathogenic yeasts (*Candida albicans*, other *Candida* spp., and *Cryptococcus neoformans*). In vivo, 200 mg/kg of body weight per day TAM reduced kidney fungal burden (-1.5 $\log_{10}$ CFU per g tissue; $P = 0.008$) in a murine model of disseminated candidiasis. TAM is a known inhibitor of mammalian calmodulin, and TAM-treated yeast show phenotypes consistent with decreased calmodulin function, including lysis, decreased new bud formation, disrupted actin polarization, and decreased germ tube formation. The overexpression of calmodulin suppresses TAM toxicity, hypofunctional calmodulin mutants are hypersensitive to TAM, and TAM interferes with the interaction between Myo2p and calmodulin, suggesting that TAM targets calmodulin as part of its mechanism of action. Taken together, these experiments indicate that the further study of compounds related to TAM as antifungal agents is warranted.

Invasive fungal infections are an increasingly common cause of morbidity and mortality among patients with compromised immune function (35). An example of this trend is the recent emergence of *Candida albicans* as one of the most common causes of bloodstream infections in hospitalized patients. Furthermore, economic estimates indicate that invasive fungal infections cost the U.S. health care system $2.6 billion annually, with antifungal drugs comprising the largest proportion of expenditures (45). Unfortunately, despite important new additions to the antifungal formulary in recent years, invasive fungal infections continue to be associated with mortality rates between 20 and nearly 100%, depending upon the infecting organism and the underlying condition of the patient (29). Consequently, the identification and development of new antifungal drugs is an important goal of current anti-infective research (44).

Recently, we reported the development of a new high-throughput screening assay to identify small molecules that cause yeast cell lysis (15). As part of the validation of this assay, we screened a library containing Food and Drug Administration-approved drugs with diverse clinical applications (1). This screen identified tamoxifen (TAM) and its structural analog clomiphene (CLM) as fungilytic molecules. In 1989, Wiseman et al. first reported that TAM has antifungal activity against *Saccharomyces cerevisiae* (46). Subsequent studies by Beggs showed that TAM is fungicidal against *Candida albicans* at concentrations between 15 and 20 μM (4).

TAM is used primarily as an estrogen receptor antagonist to treat estrogen receptor-positive breast cancer (25), but it also has antitumor activity against estrogen receptor-negative breast cancer as well other cancers, such as malignant melanomas (18), malignant gliomas (38), and lung cancers (36). Although the mechanisms of the estrogen receptor-independent effects of TAM remain a subject of debate (9), it is clear from a large body of literature that TAM has a wide range of effects on mammalian cell physiology, including antioxidant activity (47, 48), antiangiogenesis properties (43), the stimulation of transforming growth factor beta secretion (28), the induction of intracellular calcium release (14), the alteration of cellular membrane properties (47), and the induction of apoptosis (2). Consistent with this plethora of cellular effects, TAM has been shown to target a number of proteins in mammalian cells, including calmodulin, protein kinase C, phospholipase C, phosphoinositide kinase, P-glycoprotein, and swell-induced chloride channels (9). An important distinguishing factor between the estrogen receptor-dependent and -independent effects is that the estrogen receptor effects require only nanomolar concentrations of TAM, while the estrogen receptor-independent effects occur at concentrations approximately 10-fold higher (9). In the cases of protein kinase C and calmodulin, biochemical experiments have shown that TAM binds to both proteins in vitro (20, 30). Although the calmodulin binding site for TAM has not been determined experimentally, analogues of TAM have been synthesized that show increased calmodulin inhibition and decreased estrogen receptor antagonism (40).

It seems likely that the antifungal properties of TAM are related to its estrogen receptor-independent mechanisms.

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Wiseman et al. proposed that the ability of TAM to inhibit membrane peroxidation may stabilize the yeast plasma membrane and lead to growth inhibition (47). Beggs also suggested that the direct membrane-damaging effects of TAM contribute to its antifungal activity (4). More recently, Parsons et al. found that the genome-wide chemical-genetic profile of TAM in *S. cerevisiae* indicated that it interfered with calcium homeostasis (8, 34). Transcriptional profiling studies by the same group showed that TAM induces the expression of a number of genes modulated by calcineurin, a Ca^{2+}/calmodulin-dependent transcription factor that regulates the transcriptional response to calcium (34). Consistently with these observations, TAM activates a calcineurin-dependent reporter and triggers the nuclear localization of the calcineurin-dependent transcription factor Crz1p (34). These findings suggest that TAM induces an increase in intracellular calcium, a non-estrogen receptor-dependent effect previously described in mammalian cells (14). Based on these studies, it may be the case that the antifungal activity of TAM is the result of its effects on multiple physiologic processes in yeast. However, the contribution of well-characterized mammalian TAM targets such as calmodulin and protein kinase C to the antifungal activity of TAM has not specifically been investigated.

The initial characterization of the antifungal activity of TAM reported by Wiseman et al. (46) and Beggs (4) was limited to *Saccharomyces cerevisiae* and *Candida albicans* and predated the development of standardized in vitro antifungal susceptibility testing. Thus, no standardized, systematic characterization of the in vitro activity of TAM against yeast has been described to our knowledge. Similarly, the efficacy of TAM in an in vivo animal model of fungal infection has not been reported. To further characterize the antifungal activity of TAM, we initiated a study with the following goals: (i) determine the in vitro activity of TAM toward pathogenic yeast using CLSI-approved testing methods; (ii) determine if TAM has efficacy in a mouse model of disseminated candidiasis; and (iii) determine whether calmodulin or protein kinase C contributes to the antifungal activity of TAM.

As detailed below, we found that TAM is active in vitro against pathogenic yeasts, reduces kidney fungal burden in a mouse model of disseminated candidiasis, and appears to interfere with calmodulin function as part of its mechanism of action. Taken together, these data suggest that the further optimization of molecules structurally related to TAM represents a fruitful area of antifungal drug discovery research.

(This work was presented at the 48th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy/Infectious Disease Society of America 46th Annual Meeting, Washington, DC, 25 to 28 October 2008 [16].)

MATERIALS AND METHODS

Strains, media, plasmids, and antifungal drugs. The strains used in this study are listed in Table 1. All *Saccharomyces cerevisiae* strains are from the S288c genetic background. All of the strains were grown in yeast peptone dextrose (YPD) medium containing 2% (vol/vol) glucose, 2% (wt/vol) Bacto peptone (Difco Laboratories) and 1% (wt/vol) yeast extract (Difco Laboratories). YPD agar plates contained 2% (wt/vol) Bacto agar (Difco Laboratories). Unless otherwise indicated, *Candida* strains were cultivated at 37°C. *Saccharomyces cerevisiae* and *Cryptococcus neoformans* strains were cultivated at 30°C. Plasmids encoding *PKC1* (p289) and *CMD1* (pMORF-*CMD1*) were generous gifts of D. Levin (12) and E. Phizicky (11), respectively. Tamoxifen citrate and clomiphene were obtained from Sigma (St. Louis, MO) and used as received. FLC (Pfizer, New York, NY) and CAS (Merck, Rahway, NJ) were obtained from the University of Rochester Medical Center Formulary and used as received. YPD plates supplemented with tamoxifen were prepared by the addition of an appropriate amount of a dimethylsulfoxide (DMSO) solution of tamoxifen citrate (10 mg/ml) to a standard YPD agar recipe after the autoclaved mixture had cooled to approximately 60°C (1% DMSO concentration for all plates).

TABLE 1. Yeast strains

Species	Strain	Source
Saccharomyces cerevisiae	BY4741[a]	Research Genetics
Saccharomyces cerevisiae	*cmd1-226*[a]	D. Botstein (31)
Saccharomyces cerevisiae	*cmd1-228*[a]	D. Botstein (31)
Saccharomyces cerevisiae	*cmd1-231*[a]	D. Botstein (31)
Saccharomyces cerevisiae	*cmd1-239*[a]	D. Botstein (31)
Saccharomyces cerevisiae	BY4741 *MYO2-GFP*::*HIS3*	Invitrogen
Candida albicans	SC5314	E. Rustchenko laboratory collection
Candida albicans	ATCC 9028	D. Hardy
Candida tropicalis	MRO64-H	C. Haidaris laboratory collection
Candida krusei	ATCC 6258	ATCC
Candida glabrata	MRO84-R	C. Haidaris laboratory collection
Candida dubliniensis	CD-1	C. Haidaris laboratory collection
Candida parapsilosis	R058-G12	C. Haidaris laboratory collection
Cryptococcus neoformans	NYS-2	D. Hardy laboratory collection
Cryptococcus neoformans	KN99a	J. Heitman laboratory collection

[a] *Saccharomyces cerevisiae* strains are all in the S288c genetic background.

AK assay. The adenylate kinase (AK) assays were performed using a modification of our previously reported screening assay protocol (15). For *S. cerevisiae* screening, the initial inoculum was standardized by measuring the optical density (OD). An improved version of our protocol was employed for *C. albicans* and *C. neoformans*, in which the inocula were standardized by hemacytometry to give 1×10^5 cells per well. The assay otherwise was performed exactly as described in Krysan and DiDone (15).

Propidium iodide staining. Propidium iodide staining was performed as described previously (24).

Antifungal susceptibility testing. All isolates were tested in accordance with CLSI guidelines (27) using RPMI 1640 (with glutamine and phenol red, without bicarbonate) medium buffered to pH 7.0 with 0.165 M 3-(*N*-morphilino)-propanesulfonic acid, an inoculum of 1×10^3 cells/well, and incubation at 35°C. DMSO was used as the solvent for all drugs, and the final concentration was 1%. The MIC was defined as the lowest drug concentration in which no detectable growth was visible after 24 h using criteria described by CSLI (27). Testing was carried out in duplicate or triplicate for each drug and concentration on two or three separate days.

In vivo model of disseminated candidiasis. Male BALB/cAnNTac mice (10 to 12 weeks old; Taconic Labs, Germantown, NY) were treated with daily doses of either 200 mg/kg of body weight tamoxifen citrate suspended in peanut oil or a peanut oil sham via oral gavage for 7 days. Both groups then were challenged with 1.5×10^4 CFU/g by lateral tail vein injection; this dose caused a moribund state 7 to 10 days postinfection in control experiments (data not shown). For day 2 kidney organism burden determination, the animals were treated for 2 days postinfection and sacrificed. Kidneys were harvested, weighed, and homogenized in YPD. Tenfold dilutions of the homogenates were plated on YPD containing vancomycin (0.01 mg/ml) and gentamicin (0.1 mg/ml) and incubated overnight at 37°C, and then colonies were counted. Samples were plated in triplicate for each dilution. The kidney fungal burden then was calculated as CFU per gram of kidney tissue homogenized. For the experiment with survival as the endpoint, treatment was continued until sacrifice. The animals were monitored daily for morbid behavior (fur ruffling, weight loss/stature, and ability to walk) and sacrificed them when judged to be moribund. Kidneys from moribund animals were harvested and processed for kidney organism burden as described above. The

protocols for this experiment were approved by the University Committee on Animal Resources (UCAR) at the University of Rochester.

***S. cerevisiae* growth assays.** For liquid culture growth assays, BY4741 cells were precultured overnight in YPD (3 ml) at 30°C. The resulting cells were used to inoculate 25-ml cultures (final cell density, 0.1 OD_{600} units) containing YPD plus 1% DMSO, YPD plus 8 μg/ml TAM, YPD plus 1 M sorbitol, YPD plus 1 M sorbitol plus 8 μg/ml TAM, YPD plus 30 mM $CaCl_2$, and YPD plus 30 mM $CaCl_2$ plus 8 μg/ml TAM. The cultures were incubated at 30°C for 5 h, and the OD_{600} was determined. The experiment was performed in duplicate on two separate days using independent yeast colonies.

Agar plate growth assays were performed by adjusting overnight cultures of strains to a cell density of 1 OD_{600} unit. A three-step 10-fold dilution series was prepared in a flat-bottomed microtiter plate. The four different concentrations of cell suspensions were spotted on YPD or YPD + TAM plates using a pinning tool (VP Scientific, San Diego, CA). The plates were allowed to dry, incubated at 30°C for 3 to 5 days, and photographed.

The CLM and TAM MICs for *S. cerevisiae* were determined by using a published modification of the CLSI M27-A2 protocol adapted for use with BY4741 (23).

Determination of budding index. *S. cerevisiae* BY4741 cells were grown to early logarithmic phase (OD_{600}, 0.1), treated with TAM (4, 8, or 16 μg/ml) or 1% DMSO, and incubated at 30°C for 8 h. Ten percent formalin (680 μl) was added to 1 ml of culture, and the solution was incubated at 30°C for an additional 10 min. The cells were harvested by centrifugation (500 × *g* for 5 min), resuspended in phosphate-buffered saline (PBS; 240 μl), treated with 10% formalin (160 μl), and incubated at ambient temperature for 1 h. Finally, the cells were harvested by centrifugation (500 × *g* for 5 min), washed three times with PBS, and examined by light microscopy. Two hundred cells were counted for each drug concentration on three separate occasions and classified as budded or unbudded (37).

Fluorescence microscopy. *S. cerevisiae* BY4741 cells were grown to early logarithmic phase in YPD ($OD_{600} < 0.1$), treated with either TAM (8 μg/ml) or DMSO (1% final concentration), and incubated at 30°C for 8 h. The cells were fixed as described above and stained with Alexa Fluor 488-phalloidin (Invitrogen, Carlsbad, CA) by following a published procedure (37). The stained cells were analyzed by epifluorescence microscopy using a Nikon Eclipse TE-2000-E fluorescence microscope with the fluorescein isothiocyanate channel. Images were captured using MetaMorph software (Molecular Devices, Sunnyvale, CA) and processed with Adobe PhotoShop software (San Jose, CA).

S. cerevisiae BY4741 cells containing a green fluorescent protein-tagged allele of Myo2 (Myo2-GFP) were treated with 1% DMSO or 12 μg/ml TAM for 4 h. The cells were washed once with PBS, resuspended in PBS, and examined directly using the microscope and filter set described above.

Nuclear staining with 4′-6′-diamidinophenylindole (DAPI) was carried out as described previously (37).

Inhibition of germ tube formation. *C. albicans* (SC5314) cells were grown to stationary phase overnight in YPD. Aliquots (500 μl) were harvested, washed three times with PBS, and resuspended in Media 199 (10 ml) containing either 1% DMSO as a negative control or TAM (16 μg/ml). The samples were incubated for 2 h at 37°C, and the ratio of germ tubes to yeast forms was determined by light microscopy. For each experiment, 100 cells were analyzed in for both TAM and negative control samples, and the experiment was repeated on separate days with independent isolates. The propidium iodide staining (24) of samples at the time of germ tube evaluation indicated that >95% of both treated and untreated cells were viable.

In vitro calmodulin binding assay. An overnight culture of a BY4741-derived strain containing a C-terminal GFP-tagged allele of Myo2p was diluted to 0.1 OD_{600} in YPD (25 ml) and grown to mid-logarithmic phase (OD_{600}, 1.5) at 30°C. The cells were harvested by centrifugation (500 × *g*) and washed with water (25 ml). The cell pellet was resuspended in 200 μl of calmodulin binding buffer (CBB; 25 mM Tris, pH 8, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% NP-40, and 10 mM β-mercaptoethanol) containing EDTA-free protease inhibitor cocktail (Sigma, St. Louis, MO), and the cells were lysed with glass beads (150 μl) using six 20-s bursts with a Mini-Bead Beater (Biospec, Bartlesville, OK). The samples were cooled on ice for 1 min between bursts. The lysate was cleared of cellular debris by centrifugation (13,000 × *g*), and the protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). The lysate (250 μg of protein) was treated with calmodulin-Sepharose beads (25 μg; GE Healthcare) in CBB (500 μl) containing either 1% DMSO, 1% DMSO and 50 μg/ml TAM, or 1% DMSO and 50 μg/ml prochlorperazine for 1 h at 4°C. The beads were washed with CBB (four 500-μl washings) and transferred to a fresh microcentrifuge tube. Immunoprecipitates were eluted by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 μl) for 5 min, fractionated on a SDS–4 to 12% PAGE gel (Lonza, Basel, Switzerland), transferred to nitrocellulose, blocked overnight in Tris-buffered saline with 0.1% Tween (TBST) and 5% nonfat dried milk, and immunoblotted with mouse anti-GFP antibody (1:1,000 in TBST and 5% nonfat dry milk; Clontech, Mountain View, CA). The membrane then was incubated with goat anti-mouse immunoglobulin G coupled with horseradish peroxidase (1:10,000 in TBST and 5% nonfat dried milk; Bio-Rad), and protein bands were visualized by chemiluminescence (ECL Plus kit; Amersham, Piscataway, NJ). Relative amounts of Myo2-GFP immunoprecipitated by calmodulin-Sepharose were estimated by densitometry and normalized to the DMSO-treated lanes.

Statistical analysis. In vitro data were analyzed by Student's *t* test. The kidney tissue burden was analyzed by Mann-Whitney U test. $P < 0.05$ was considered indicative of a statistically significant difference between two data sets.

RESULTS

TAM and CLM disrupt yeast cell integrity. TAM and CLM were identified as fungilytic molecules in a screen for such molecules using *S. cerevisiae* (15). To confirm that TAM and CLM also caused lysis in other species of yeast, we measured AK release from *C. albicans* (strain SC5314) and *Cryptococcus neoformans* (strain NYS-2) at drug concentrations equal to the MIC (see below). AK is an intracellular enzyme that is released into the culture medium when the integrity of the yeast cell is disrupted. As we have recently described, extracellular AK activity is a sensitive reporter of yeast cell lysis using an assay kit (ToxiLight; Lonza) that couples AK-catalyzed ATP production with luciferase activity (15). Logarithmic-phase SC5314 and NYS-2 cells were treated with TAM (32 μg/ml for SC5314 and 64 μg/ml for NYS-2), CLM (32 μg/ml for SC5314 and 64 μg/ml for NYS-2), or vehicle (DMSO) for 5 h, and extracellular AK activity was determined using a modification of our previously reported protocol (15). As shown in Fig. 1B, TAM and CLM both induce increased extracellular AK activity (~60-fold) at levels comparable to that observed with *S. cerevisiae* (15). Similarly, TAM and CLM induced AK release in *C. neoformans*. The background amount of extracellular AK is approximately twofold higher in *C. neoformans* and, consequently, the higher absolute amount of AK in the medium of treated *C. neoformans* cells relative to *C. albicans* corresponds to a nearly identical 60-fold increase in AK release. Consistently with our previous studies with the AK assay (15), propidium iodide staining confirmed that TAM- and CLM-treated SC5314 and NYS-2 cells lost cellular membrane integrity under the assay conditions (Fig. 1C). These data indicate that TAM and CLM disrupt yeast cell integrity and are consistent with Beggs' finding that TAM is fungicidal toward *C. albicans* (4).

TAM and CLM are active in vitro against a range of pathogenic yeast. To our knowledge, the in vitro activities of TAM and CLM have not been evaluated using standardized CLSI susceptibility testing methods. To test the scope of TAM and CLM antifungal activity, we determined MICs for these two drugs against a set of common *Candida* species as well as *C. neoformans* according to CLSI protocol M27-2A (Table 2). The MIC of TAM against *Candida* species ranged from 8 μg/ml for *C. glabrata* to 64 μg/ml for *C. parapsilosis*. The MIC was 32 μg/ml for two standard reference strains of *C. albicans*. Interestingly, *S. cerevisiae* (MIC, 12 μg/ml) was more susceptible than *C. albicans* (MIC, 32 μg/ml) and was similar to *C. glabrata* (MIC, 8 μg/ml), a finding consistent with their close evolutionary relationship (3). Consistent with its fungilytic activity toward *C. neoformans*, TAM also showed in vitro activity

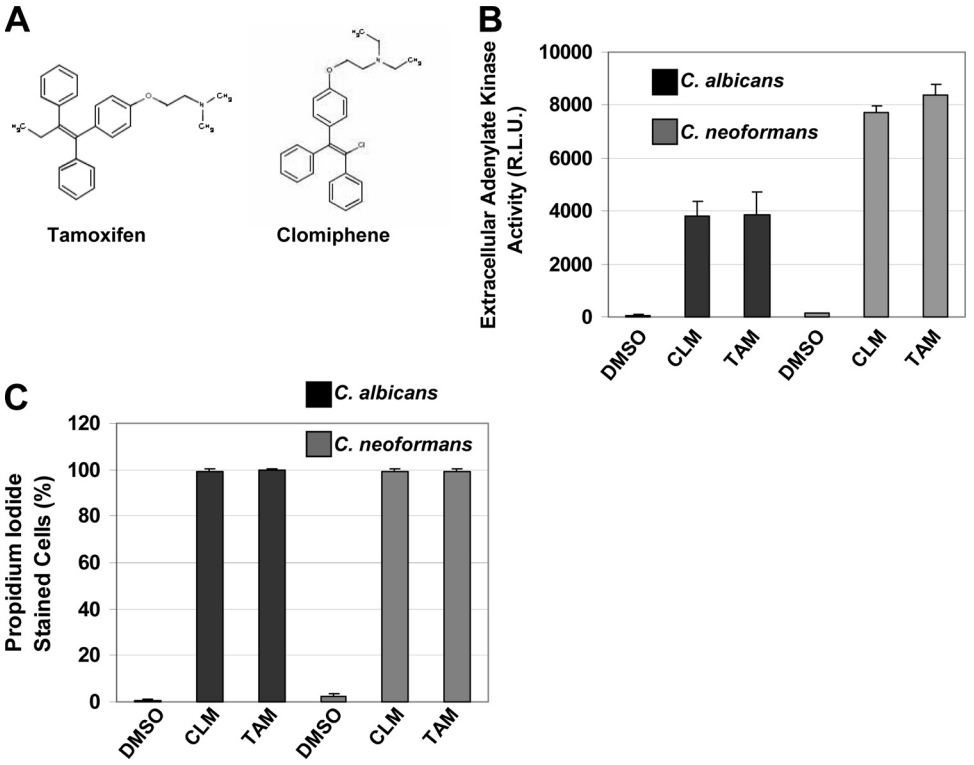

FIG. 1. Tamoxifen and clomiphene disrupt yeast cell integrity. (A) Chemical structure of TAM and CLM. (B) TAM and CLM causes the release of AK into culture medium in *C. albicans* (SC5314) and *C. neoformans* (NYS-2). Yeast cells were treated with TAM (SC5314, 32 μg/ml; NYS-2, 64 μg/ml) or CLM (SC5314, 32 μg/ml; NYS-2, 64 μg/ml) for 5 h and processed for extracellular AK activity as described in Materials and Methods. Mean relative light units (R.L.U.) and standard deviations (error bars) from three independent experiments performed in triplicate are shown. (C) TAM and CLM treatment of SC5314 and NYS-2 cells causes cell death by propidium iodide staining. SC5314 and NYS-2 cells were treated with TAM and CLM as described for panel B and processed for propidium iodide staining as previously described (24). Three independent experiments (100 cells per experiment) were conducted for each treatment. The percentage of cells stained by propidium iodide was calculated for each experiment. Bars indicate the means, and error bars indicate standard deviations.

(MIC, 64 μg/ml) toward this species, although it was among the least susceptible species. The activity of CLM was within a single twofold dilution of TAM except against *C. glabrata*, for which it was fourfold less active. Thus, TAM and CLM show in vitro activity against pathogenic yeast.

TAM treatment reduces kidney organism burden in a mouse model of disseminated candidiasis. To further test the potential of TAM and related molecules as antifungal drugs, we examined the efficacy of TAM treatment in a model of disseminated candidiasis using immunocompetent mice. The pharmacodynamics/pharmacokinetics of TAM have been studied in mice, and we used those data to guide our dosing regimen for the experiment (39); please note that we did not measure serum or tissue TAM levels in the animals used in our experiment. TAM is metabolized in mice and humans to 4-hydroxy-TAM and *N*-desmethyl-TAM (25, 39). All three compounds are active estrogen receptor antagonists, but the antifungal properties of the metabolites have not been investigated to our knowledge (25). Robinson showed that prolonged (7-day) administration of TAM (200 mg/kg) to mice provided serum levels of 4 to 5 μM for TAM, 4-hydroxy-TAM, and *N*-desmethyl-TAM (39); these serum levels are similar to those reported for humans receiving high doses (200 to 400 mg/day) of TAM during experimental therapy of cancer (38).

TAM also is concentrated in tissues (brain, liver, kidney, and uterus) in mice and humans to levels 100-fold greater than those of serum levels (25, 36, 39). Based on these considerations, the 7-day pretreatment regimen of 200 mg/kg/day could give up to ~400 μM TAM (~160 μg/ml) in the kidneys of mice, levels well above the MIC we observed for *C. albicans* (32 μg/ml). Thus, male BALB/cAnNTac mice were treated with 200 mg/kg tamoxifen citrate ($n = 5$) or peanut oil carrier

TABLE 2. MICs of TAM for *S. cerevisiae*, *Candida* spp., and *C. neoformans*[a]

Species	Strain	MIC (μg/ml)	
		TAM	CLM
Saccharomyces cerevisiae	BY4741	12	18
Candida albicans	ATCC 90028	32	>64
Candida albicans	SC5314	32	32
Candida parapsilosis	R058-G12	64	64
Candida dubliniensis	CD1	16	32
Candida glabrata	MR084-G12	8	32
Candida tropicalis	MR064-H	32	64
Cryptococcus neoformans	NYS 3-81	64	64
Cryptococcus neoformans	KN99 α	64	64

[a] Data are based on at least two independent replicates performed on separate days in triplicate. All replicates of MIC determinations were within a twofold dilution of the reported value. MIC endpoints are defined as no turbidity by visual inspection by following the CLSI protocol (27).

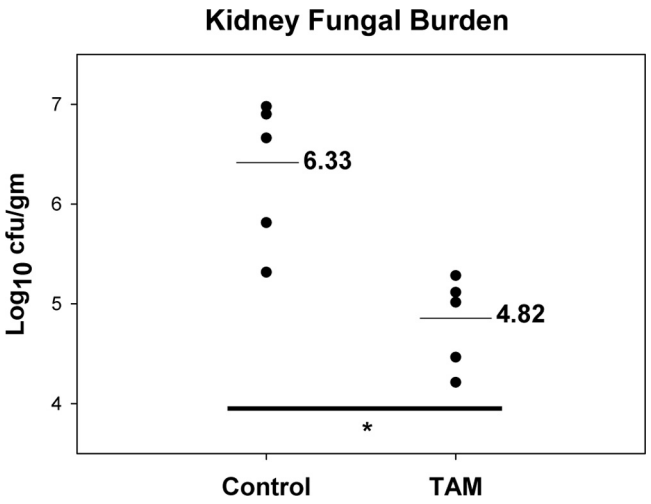

FIG. 2. TAM treatment decreases kidney fungal burden on day 2 postinfection in a murine model of disseminated candidiasis. Mice were treated with TAM (200 mg/kg; $n = 5$) or vehicle (peanut oil; $n = 5$) by oral gavage for 7 days, injected with *C. albicans* SC5314 (1.5×10^4 CFU/g) by the lateral tail vein, and treated for 2 days before sacrifice. The kidney fungal burden was determined as described in Materials and Methods and expressed as $\log_{10}$ CFU per gram of tissue. The points of the graph represent individual animals in each group, and the horizontal line indicates the means for the group as a whole. Differences between groups were analyzed by Mann-Whitney U test (*, $P = 0.008$).

($n = 5$) orally for 7 days prior to challenge with *Candida albicans* by tail vein injection (SC5314 inoculum, 1.5×10^4 CFU/g body weight). TAM and placebo then were administered for two additional days. At that time, the animals were sacrificed and the kidney organism burden was determined according to the procedure described in Materials and Methods. As shown in Fig. 2, the kidney organism burden was ~1.5 $\log_{10}$ CFU/g of kidney tissue lower for TAM-treated mice than for sham-treated mice ($P = 0.008$ by Mann-Whitney U test). The level of kidney organisms in the sham treatment group (6.44 $\log_{10}$ CFU/g kidney tissue) was similar to that reported by other groups (13), indicating that this protocol generates a significant infection.

A separate experiment using the same protocol but with survival as the outcome showed no difference between TAM and sham treatment groups (data not shown). This experiment was complicated by the fact that the mice showed signs of toxicity similar to those reported by Robinson et al. when micromolar serum and tissue levels were attained (39); we note that this supports our expectation that micromolar levels of TAM were achieved by this dosing regimen in the mice used in our experiment. Although there was no effect on mortality, the kidney burden (see Fig. S1 in the supplemental material) was significantly lower in the TAM-treated group at the time of euthanasia (6.44 $\log_{10}$ CFU/g for TAM and 5.90 $\log_{10}$ CFU/g for sham; $P = 0.029$ by Mann-Whitney U test). These experiments indicate that TAM has in vivo activity against *C. albicans* and further support the potential utility of this class of molecules as antifungal drugs.

Calmodulin modulates TAM toxicity in *S. cerevisiae*. As discussed in the introduction, TAM has a variety of non-estrogen receptor-mediated effects on eukaryotic cells, with the inhibition of protein kinase C and calmodulin being particularly well-characterized examples. Homologues of protein kinase C (*PKC1*) and calmodulin (*CMD1*) are essential in both pathogenic yeast and the model yeast *S. cerevisiae* (7, 12, 17). Therefore, we wondered whether the inhibition of these proteins in yeast contributed to the antifungal activity of TAM. The overexpression of candidate target genes in yeast is a useful approach for identifying the potential mechanisms of action for antifungal molecules (21, 22). We therefore took a genetic overexpression approach to test the hypothesis that the inhibition of *PKC1* or *CMD1* is related to the mechanism of the antifungal activity of TAM.

The TAM sensitivity of *S. cerevisiae* cells (BY4741) harboring multicopy plasmids encoding *PKC1* (12) or *CMD1* (11) was compared to that of the parental strain transformed with the corresponding empty vectors (Fig. 3A and B). Consistently with previous reports (11, 12), the overexpression of neither *PKC1* nor *CMD1* affected growth under standard growth conditions (30°C). As is frequently the case, higher concentrations of TAM were required to suppress the growth of the reference strain (BY4741) on agar plates compared to that of liquid culture. The overexpression of *PKC1* actually increased the sensitivity of *S. cerevisiae* to TAM (Fig. 3A), while the increased expression of *CMD1* suppressed the toxicity of TAM (Fig. 3B). Although this experiment is by no means definitive, it is consistent with TAM targeting calmodulin-related cellular processes and is inconsistent with the inhibition of *PKC1* as a mechanism of the antifungal activity of TAM.

To further test the hypothesis that TAM inhibits calmodulin in yeast, we examined the effect of a set of hypofunctional calmodulin mutants on TAM sensitivity. Calmodulin is an essential gene in yeast and performs four well-defined, essential functions related to (i) the actin cytoskeleton; (ii) calmodulin localization; (iii) nuclear division; and (iv) bud emergence (7). Ohya and Botstein (31) isolated a library of conditional, separation-of-function *cmd1* mutants that specifically affect each of the four essential calmodulin functions in *S. cerevisiae* (Fig. 3C). If TAM interferes with calmodulin function, then strains with mutations in calmodulin would be predicted to have increased sensitivity to TAM. As shown in Fig. 3D, all four classes of *cmd1* mutants are hypersensitive to TAM compared to a wild-type strain with the identical genetic background. The calmodulin mutant defective for bud emergence (*cmd1-231*) was the most severely affected. These experiments further support the possibility that TAM interferes with cellular processes related to calmodulin as part of its mechanism of action.

TAM treatment interferes with polarized growth in yeast. To gain further insight into the mechanism of action for TAM, we performed a set of assays to determine if TAM-treated cells displayed phenotypes characteristic of calmodulin mutants. Since some of the phenotypes displayed by calmodulin mutants in yeast can be suppressed by increased extracellular calcium (10, 32), we asked if increased extracellular calcium modulated the effects of TAM. Indeed, the addition of supplemental calcium to the growth medium significantly increased the growth of TAM-treated cultures (Fig. 4A). In contrast, the addition of sorbitol as an osmotic support did not affect TAM toxicity, suggesting that the effect was specific to calcium and was not due to osmotic stabilization. Since the addition of sorbitol to

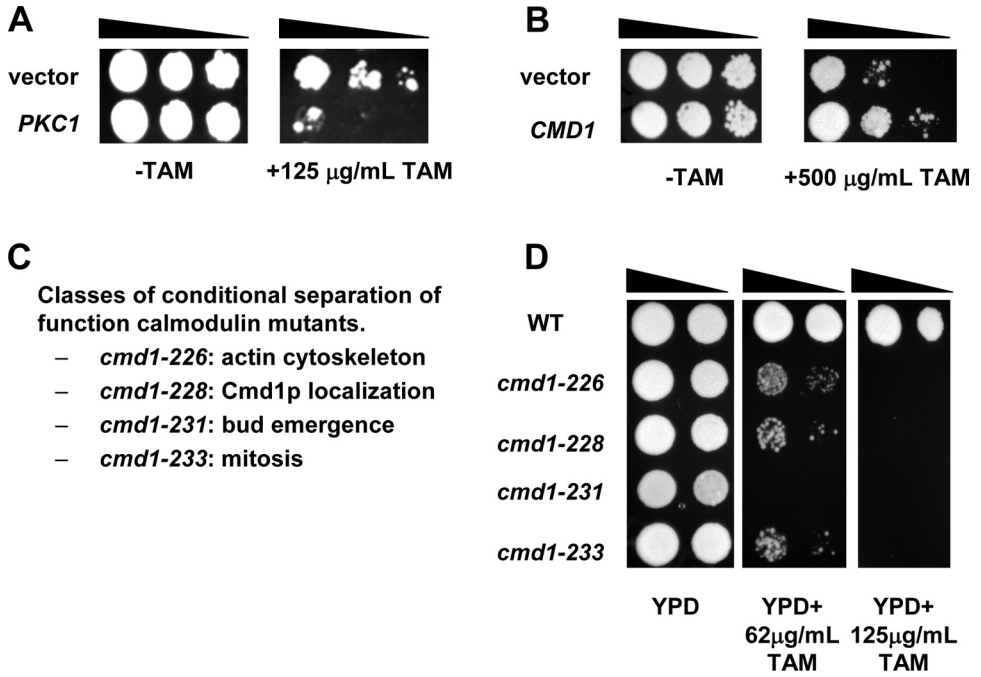

FIG. 3. Calmodulin modulates TAM toxicity in *S. cerevisiae*. (A and B) A 10-fold dilution series of BY4741 cells transformed with a multicopy plasmid (2μ) expressing *PKC1* (A), *CMD1* (B), or empty vector were spotted on YPD plates containing either 1% DMSO or TAM at the indicated concentrations, incubated at 30°C for 3 days, and photographed. (C) Functional classes of the calmodulin separation of function mutants (31). (D) The indicated calmodulin mutants and wild-type cells (WT; S288c genetic background) were spotted on TAM containing plates as described for panels A and B. For each phenotype, independent isolates of each strain were tested in duplicate, and a representative experiment is shown.

the growth medium decreases the toxicity of protein kinase C inhibitors (50) and suppresses the lethality of the *pkc1*Δ mutation in yeast (19), the fact that sorbitol does not affect TAM toxicity is consistent with our genetic data suggesting that protein kinase C inhibition does not contribute significantly to the antifungal activity of TAM.

Because the bud emergence *cmd1* mutant (*cmd1-231*) was the most susceptible to TAM, we examined the budding index of *S. cerevisiae* cells over a range of TAM concentrations to determine if TAM affected new bud formation. As shown in Fig. 4B, TAM treatment increased the proportion of unbudded cells relative to that of untreated cells, indicating that TAM interferes with bud emergence or polarized growth. A second characteristic of *cmd1-231* mutants is that unbudded cells accumulate multiple nuclei when incubated at the restrictive temperature (31). We therefore stained TAM-treated and untreated cells with DAPI and determined the proportion of unbudded cells with multiple nuclei. While none of the untreated cells showed abnormal nuclear staining ($n = 600$ for each group), 15% of TAM-treated, unbudded cells showed more than one nuclei (see Fig. S2 in the supplemental material). These data strongly suggest that TAM treatment interferes with new bud formation and provide further support for the notion that TAM-treated yeast phenocopy hypofunctional calmodulin mutants. Since the loss of protein kinase C activity leads to an accumulation of small budded cells (19), these data are inconsistent with protein kinase C inhibition contributing to TAM toxicity in yeast.

To further explore the effect of TAM treatment on polarized growth, we examined the actin cytoskeletal structure of TAM-treated cells. The Alexa Fluor-phalloidin staining of TAM-treated cells showed that the actin cytoskeleton does not polarize to new bud sites (37). Instead, TAM-treated cells display extensively delocalized actin patches as well as a paucity of actin cables (Fig. 4C). It therefore appears that TAM treatment not only blocks new bud emergence but also interferes with the polarization of the actin cytoskeleton. *cmd1-226* also displays a delocalized actin cytoskeleton (31) and, thus, TAM treatment phenocopies a second *cmd1* mutant.

We also examined TAM-treated cells for phenotypes consistent with *cmd1-228*, which specifically affects Cmd1p localization, and *cmd1-231*, which has defects in mitosis (31), but we did not consistently observe significant phenotypes characteristic of these mutants (data not shown). Therefore, it appears that TAM treatment affects polarized growth and new bud emergence and, thus, shows phenotypes consistent with the loss of some, but not all, calmodulin functions.

Since our data indicate that TAM interferes with polarized growth in yeast, we hypothesized that it may inhibit germ tube formation in *C. albicans*, a process related to virulence and dependent on the calmodulin-binding protein Myo2p (49). As shown in Fig. 4D, treatment with subinhibitory concentrations of TAM (16 μg/ml) significantly decreases the ability of *C. albicans* cells to form germ tubes. Importantly, this effect does not appear to be due to the fungicidal effects of TAM, because >95% of treated and untreated cells were viable under the conditions of the experiment, as determined by propidium iodide staining (24). Since the yeast-to-hypha transition is required for *Candida albicans* virulence and its inhibition has been proposed as a possible treatment strategy (41), these data

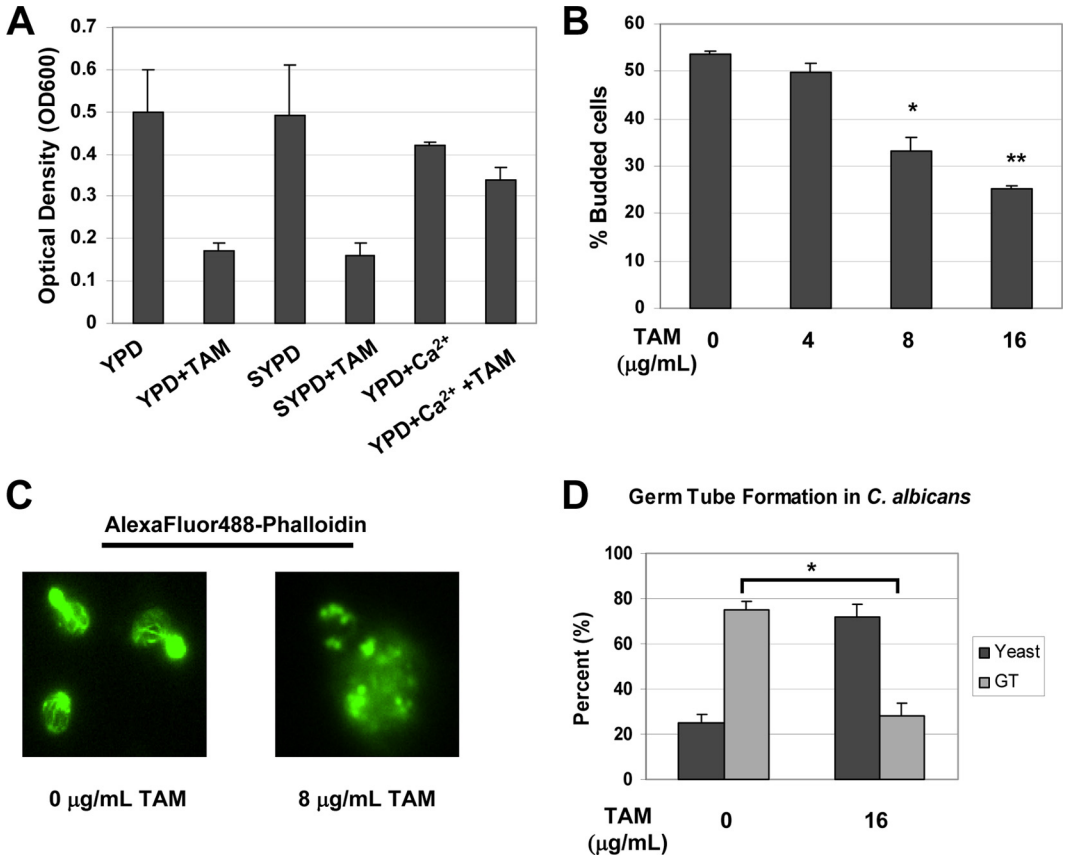

FIG. 4. TAM affects calcium dependent processes and polarized growth. (A) Extracellular calcium, but not sorbitol, suppresses TAM toxicity. Cultures (25 ml; 0.1 OD_{600} unit) of *S. cerevisiae* (BY4741) in YPD supplemented with either 30 mM $CaCl_2$ or 1 M sorbitol (SYPD) were treated with TAM (8 μg/ml) or 1% DMSO solvent and incubated for 5 h at 30°C, and growth was assessed according to the OD_{600}. Means and standard deviations (error bars) from three separate experiments are shown. (B) TAM inhibits bud formation in *S. cerevisiae*. Logarithmic-phase BY4741 cells in YPD (0.1 OD_{600} unit) were treated with the indicated concentrations of TAM, incubated for 5 h at 30°C, and fixed with formalin, and the percentage of budded cells was determined by light microscopy. Mean percentages of budded cells from three separate experiments (100 cells/experiment) are presented. Error bars indicate standard deviations. (C) TAM causes actin depolarization in *S. cerevisiae*. BY4741 cells were treated with the indicated concentrations of TAM or 1% DMSO, fixed, and stained with Alexa Fluor 488-phalloidin to visualize actin. (D) TAM inhibits germ tube (GT) formation. Germ tubes were induced in *C. albicans* (SC5314) cells with and without TAM (2 h of incubation). The percentage of germ tubes and yeast was determined by light microscopy. The means of three independent experiments of 100 cells are shown, with error bars indicating standard deviations (*, $P < 0.005$ by Student's *t* test). Propidium iodide staining indicated that >95% of cells were viable at the time of germ tube assessment.

indicate that TAM kills yeast and prevents a key morphological transition that is associated with virulence.

TAM interferes with binding of Myo2p to calmodulin in vitro. Since TAM toxicity appears to be at least partly modulated by calmodulin and TAM-treated cells show phenotypes consistent with defects in calmodulin-mediated processes, we next tested whether TAM interfered with the binding of calmodulin to its cellular targets. To do so, we carried out an in vitro binding experiment using calmodulin-Sepharose beads and lysates of a *S. cerevisiae* strain harboring a GFP-tagged allele of *MYO2*, an essential calmodulin binding protein involved in a variety of cellular processes related to polarized growth (6, 52). Total cell lysates from *MYO2-GFP* cells were treated with calmodulin beads in the presence of DMSO carrier, TAM (50 μg/ml), or the known calmodulin inhibitor prochlorperazine (50 μg/ml) (14). The immunoprecipitates were analyzed by Western blotting using anti-GFP antibody. As shown in Fig. 5A, the presence of TAM decreased the amount of Myo2-GFP bound to calmodulin beads relative to that of untreated beads. Prochlorperazine also decreased binding but to a lesser extent than TAM. The semiquantitative analysis of the effect of TAM and prochlorperazine on Myo2-GFP binding to calmodulin showed that TAM decreases the amount of Myo2-GFP immunoprecipitated under these conditions by approximately threefold (Fig. 5B).

Since Myo2p localization is dependent upon its interaction with calmodulin (6), we also examined the effect of TAM on the localization of Myo2-GFP by fluorescence microscopy. Myo2p localizes to the incipient bud site early in the cell cycle and, as shown in Fig. 5C, to the actomyosin ring in medium-to-large budded cells at the time of cytokinesis (6). Consistently with the inhibitory effect of TAM on Myo2p-calmodulin interactions in vitro, Myo2-GFP localization is disrupted by TAM treatment. In TAM-treated cells containing Myo2-GFP expressed from its endogenous promoter, Myo2p fails to form the bar-like structure at the bud neck in cells with medium-

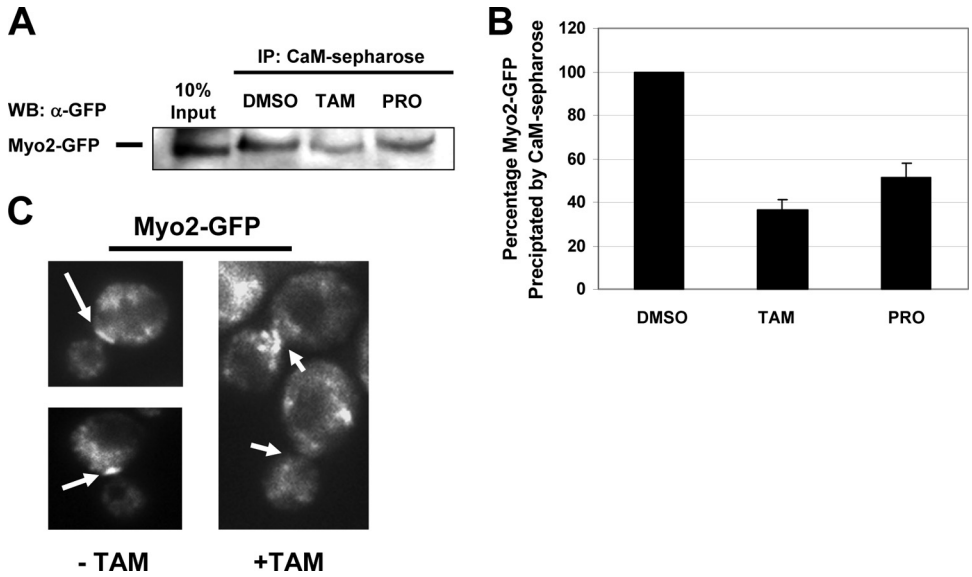

FIG. 5. TAM interferes with the in vitro binding of Myo2p to calmodulin and the cellular localization of Myo2p. (A) Cell lysates of *S. cerevisiae* BY4741 containing Myo2-GFP were immunoprecipitated (IP) with calmodulin-Sepharose beads in the presence of 1% DMSO carrier, TAM (50 μg/ml), or the bona fide calmodulin inhibitor prochlorperazine (PRO; 50 μg/ml). Immunoprecipitates were eluted from beads with SDS-PAGE loading buffer and analyzed by Western blotting (WB) with anti-GFP antibodies. (B) Semiquantitative analysis of the effect of TAM and PRO on Myo2-GFP immunoprecipitation by calmodulin-Sepharose. The amount of Myo2-GFP precipitated by calmodulin-Sepharose was estimated by the densitometry of the bands visualized by Western blotting, of which the blot in panel A is a representative example. The amount precipitated in the presence of 1% DMSO was set at 100%. Bars represent the means of three independent experiments, and error bars indicate standard errors of the means. (C) *S. cerevisiae* BY4741 cells containing Myo2-GFP were treated with 1% DMSO or 12 μg/ml TAM for 4 h and analyzed by fluorescence microscopy.

sized buds (Fig. 5C). Because TAM treatment delocalizes Myo2-GFP throughout the cytoplasm, we could not assess its effect on the localization of Myo2-GFP during new bud formation, as it was not possible to distinguish delocalized Myo2-GFP from Myo2-GFP at the incipient bud site (data not shown). Taking these results together, the ability of TAM to interfere with the binding of calmodulin to a protein involved in polarized growth (Myo2p) and the mislocalization of Myo2-GFP in TAM-treated cells are consistent with the hypothesis that calmodulin inhibition contributes to the antifungal activity of TAM.

DISCUSSION

Tamoxifen displays both in vitro and in vivo activity against pathogenic yeast. Although the antifungal activity of TAM was described 20 years ago, no systematic in vitro or in vivo analysis of this activity had been reported prior to our study. In vitro susceptibility testing clearly shows that TAM and CLM have activity toward pathogenic yeasts, with MICs of 8 to 64 μg/ml for TAM. Consistently with its fungicidal activity, we observed no evidence of trailing endpoints. The in vitro activity of these molecules seems sufficient to support the further evaluation of other examples of this class as antifungal drugs.

TAM significantly decreased kidney fungal burdens in a mouse model of disseminated candidiasis, indicating that TAM has in vivo activity against *C. albicans*. However, no difference between TAM- and sham-treatment groups was found when survival was used as the endpoint (data not shown). The interpretation of this experiment was somewhat complicated by the fact that the treatment of mice with TAM for 10 to 14 days caused evidence of toxicity (weight loss and ruffling), a phenomenon also reported by Robinson et al. (39). In humans, much higher serum levels are achievable with no apparent increase in side effects relative to standard doses (38), suggesting that the toxicity observed in mice is due to physiologic differences between mice and humans. Indeed, the experimental therapy of aggressive brain tumors using high-dose tamoxifen regimens have shown that micromolar (~10 to 20 μg/ml) serum TAM concentrations are safely tolerated for as long as 6 months. Such serum levels are quite close to the MICs we observed for TAM and, consequently, suggest that further structure-activity or structure-pharmacodynamic optimization of this general structural class of molecules will lead to a clinically useful candidate.

Note that we did not explicitly measure serum or tissue TAM levels in the mice receiving TAM, and we relied on previous studies of the pharmacodynamics/kinetics of TAM in mice to choose the dosing regimen (39). Since there was a significant decrease in kidney fungal burden and because we observed signs of TAM toxicity associated with micromolar serum and tissue levels in mice (39), it seems likely that tissue levels close to the TAM MIC for *C. albicans* were achieved in the TAM-treated mice.

Recently, the concept of treating *C. albicans* infections with molecules that inhibit the yeast-to-hypha transition has received attention (41, 42). For example, a high-throughput screen for molecules that decrease hypha formation identified a set of compounds that are active at micromolar concentrations (42). Our experiments indicate that TAM has activity as an inhibitor of germ tube formation at similar concentrations,

and thus it is possible that the ability of TAM to inhibit germ tube formation at sub-growth-inhibitory concentrations contributed to the decreased fungal burdens we observed. These considerations raise the interesting possibility that molecules structurally related to TAM offer two mechanisms for the treatment of yeast infections: (i) the direct killing of the organism and (ii) the inhibition of virulence. Taken together, these in vitro and in vivo data support the idea that further structure-activity studies of molecules related to TAM will provide potentially useful antifungal compounds.

Tamoxifen appears to target processes related to calmodulin in yeast. As outlined in Introduction, TAM has a wide range of physiologic effects on eukaryotic cells (9). Previous studies of the mechanism of the antifungal activity of TAM had identified membrane perturbation (47) and interference with calcium homeostasis (34) as possible modes of action. Since a number of studies (9) in mammalian cells clearly showed that TAM targets protein kinase C and calmodulin, and since the homologs of both proteins are essential in yeast (7, 12), we wondered if the inhibition of either of these targets contributes to the antifungal activity of TAM. Our genetic, phenotypic, and biochemical data indicate that calmodulin, but not protein kinase C, is involved in the antifungal activity of TAM. Although the ability of TAM to disrupt the interaction of calmodulin with its binding partner Myo2p supports a possible direct effect of TAM on calmodulin, it also is possible that TAM interferes with processes that require calmodulin function as a compensatory response. Indeed, it seems unlikely, given the plethora of cellular effects caused by TAM, that a single mode of action is responsible for the antifungal activity of TAM.

With respect to the possibility that TAM exerts antifungal effects through multiple mechanisms, our results provide a possible explanation for an observation made by Parsons et al. during their chemical-genetic studies of TAM (34). They observed that both TAM and amiodarone (AMD), an antiarrhythmic molecule with antifungal activity, gave chemical genetic profiles indicative of molecules that interfere with calcium homeostasis (34). Consistently with that finding, TAM and AMD induce the activation of a calcineurin-dependent reporter construct and cause the nuclear localization of Crz1p, a calcineurin-regulated transcription factor (34, 51). These observations are consistent with TAM and AMD causing an increase in intracellular calcium, effects previously described for mammalian cells (14). Meund and Rao have extensively studied the mechanism by which AMD exerts antifungal activity and found that it induces increased calcium spikes in yeast cells (26). Although the chemical-genetic data of Parsons et al. suggested that AMD and TAM have similar mechanisms of action in yeast, they also noted that AMD induced a fivefold larger increase in calcineurin-dependent signaling than TAM (34). Correspondingly, the percentage of cells with Crz1p localized to the nucleus was twofold lower in TAM-treated cells than in amiodarone-treated cells (34). We observed that TAM-treated cells arrest as unbudded cells in G_1 (Fig. 4B), while Zhang and Rao have shown that AMD-treated cells arrest at the G_2-M stage of the cell cycle (51). Thus, it appears that TAM and AMD share some mechanistic characteristics but also have distinct effects on yeast cell physiology.

We further suggest that the decreased calcineurin signaling in the TAM-treated cells is due to the ability of TAM to interfere with calmodulin. Calcineurin is a calmodulin-dependent phosphatase (7, 33), and therefore, as part of a multimechanism mode of action, TAM may increase the level of intracellular calcium through one mechanism and concurrently interfere with the cell's ability to compensate for the increased calcium by partially inhibiting calcineurin activation. Although somewhat speculative, this model is consistent with our data, the observations of other groups, and the effects of TAM on mammalian cells.

In summary, our studies of the antifungal activity of TAM and CLM indicate that they represent a potentially useful class of compounds for further antifungal drug discovery and development. Two attractive features of this class are its high oral bioavailability and fungicidal activity. Furthermore, structure-activity studies performed during the development of this class of molecules as breast cancer therapies may provide a preexisting set of molecules that could be screened as an expedient approach to the identification of analogues with improved antifungal or pharmacologic properties.

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